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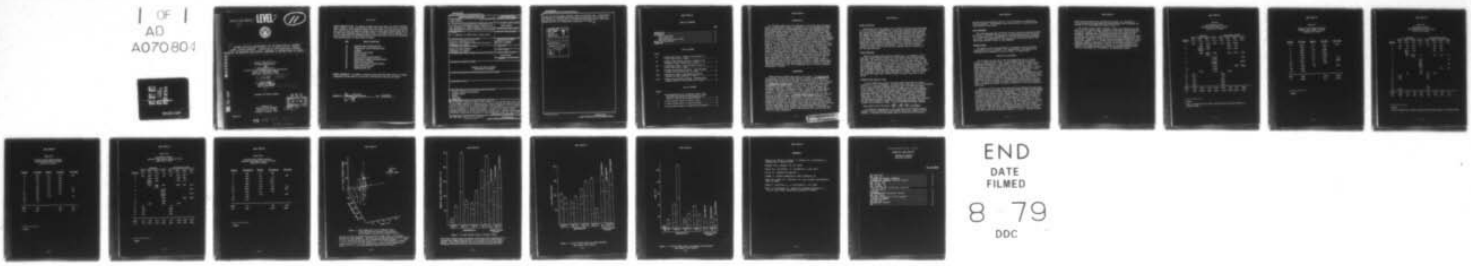

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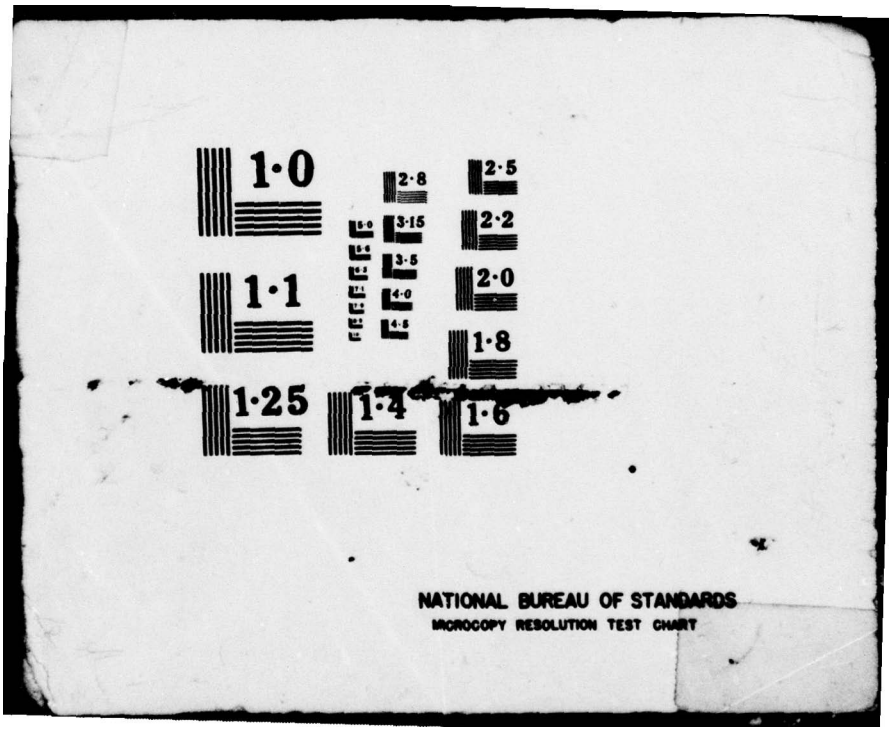
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LEVEL II

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URINARY MOLECULAR DETERMINANTS FOR THE PREDICTION OF ISCHEMIC ANOXIC STRESS PATHOLOGY: EVALUATION OF LIPID PEROXIDES, PHENOLIC ACIDS AND GENERATED FREE RADICAL COMPOUNDS AS BIOINDICATORS OF STRESS.

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15 May 1979

29 p.

PHASE REPORT

AIRTASK NO. ZR04106
Work Unit No. GC135

ZR0400I

APPROVED FOR PUBLIC RELEASE

Prepared for
OFFICE OF NAVAL RESEARCH
Department of the Navy
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1. REPORT NUMBER NADC-79084-60	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Urinary Molecular Determinants for the Prediction of Ischemic Anoxic Stress Pathology: Evaluation of Lipid Peroxides, Phenolic Acids and Generated Free Radical Compounds as Bioindicators of Stress		5. TYPE OF REPORT & PERIOD COVERED PHASE REPORT 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) H. W. Shmukler, B. David Polis, Elaine Soffer		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Naval Air Development Center Aircraft & Crew Systems Technology Directorate Warminster, PA 18974		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS AIRTASK NO. ZR04106 Work Unit No.GC135
11. CONTROLLING OFFICE NAME AND ADDRESS Office of Naval Research Department of the Navy Arlington, VA 22217		12. REPORT DATE 13. NUMBER OF PAGES 17
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Urinary Molecular Determinants
Ischemic Anoxic
Stress
Pathology
Prediction

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In preliminary studies in which a small number of test subjects were used, the 24-hr urinary excretion of phenolic acids, lipid peroxides and generated free radicals when plotted in a three-dimensional manner appeared to be a bioindicator of the effects of pathological stress. This methodology was tested with larger populations of control, acceleration stressed, and coronary artery diseased subjects to obtain statistically reliable data. Results showed large individual variations in excretion of three parameters within each test group,

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as well as for individual subjects tested on different days. Consequently, overlapping of calculated mean values and standard error of the means, for the test groups, invalidates use of GFR-lipid peroxide-phenolic acid three-dimensional plot as a noninvasive bioindicator of stress.

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INTRODUCTION

In a previous report from this laboratory it was shown that the plasma levels of phosphatidyl glycerol, phosphatidic acid and phosphatidyl ethanolamine when plotted in a three-dimensional manner could differentiate not only stressed human populations from normals, but also different stressed populations from each other; ie, combat flying, acceleration stress, sleep deprivation, schizophrenia.[1] The importance of this work was that it demonstrated the feasibility of finding a molecular bioindicator or bioindicators of stress in body fluids. Unfortunately the complexity and the time consuming nature of the analytical methodology[2,3] precludes its use as a routine diagnostic tool. In addition, the requirement for blood plasma introduced a possible variable resulting from subject resistance of intolerance to blood sampling. To overcome these objections and difficulties, studies were undertaken to develop a noninvasive rapid analytical technique for molecular correlates of stress; for this purpose urine was chosen. In preliminary studies a number of analytical methodologies were screened. Of these, the levels of (a) generated free radicals (GFR) in urine extracts (b) phenolic acids in whole urine, and (c) lipid peroxides in whole urine when plotted in a three-dimensional manner appeared to differentiate severely stressed human populations from normal and mildly stressed populations.[4] This report describes the extension of the above studies to larger numbers of test subjects, normal as well as stressed, in order to obtain statistically significant results and thus define unequivocally the efficacy of the GFR-lipid peroxide-phenolic acid three-dimensional plot as a valid noninvasive bioindicator of stress.

EXPERIMENTAL

Three groups of subjects were used in this study: (a) Control group (16 subjects) consisting of normal healthy civilian and NAVAIRDEVCEN aircrewmen varying in age from 25-60 years (one female); most of the naval aircrewmen also served as test subjects for the acceleration stress studies; (b) Acceleration stressed group[5] (13 subjects) consisted of young healthy NAVAIRDEVCEN aircrewmen between 23 and 35 years of age capable of withstanding acceleration loading to at least + 3.0 G_z without nausea and peripheral light loss. Acceleration study 1 consisted of 5 males exposed to grayout. Acceleration studies 2, 3 and 4 groups (14 subjects) were exposed to low level + G_z for short time periods of 2-3 + G_z, 2-3 + G_z and 3-4 + G_z respectively;[5] (c) Coronary artery disease group (20 subjects) consisted of male and female hospitalized (Presbyterian-University of Pennsylvania Medical Center, Philadelphia, PA) patients awaiting coronary artery bypass operations, who served as the severely stressed group. This designation was based on the premise that because of their severe physical disability (cardiac artery disease) coupled with the mental anxiety resulting from the uncertainty of the outcome of the impending operation, these patients experienced severe physical and mental trauma. In addition post-operative urines from four cardiac bypass patients were collected and also tested for stress parameters.

URINE COLLECTIONS

Twenty-four hour urine samples were collected in plastic bags containing 0.5 ml of toluene as a preservative. During the collection period the bags were stored at room temperature. When the urine collection was completed, the samples were processed immediately or stored frozen at -20° until used. The 24-hour urine samples from the control group were collected starting in the morning. In the acceleration studies, urine samples were collected 24 hours before and after exposure to acceleration stress. The urine collected prior to acceleration stress served as an individual control urine. Except for the prohibition of aspirin ingestion, no dietary or activity restrictions were placed on the subjects. In the case of the cardiac patients no restrictions of any kind were imposed; however, medication if any was used was noted.

URINE EXTRACTION

500 ml of filtered whole urine was passed over 1 in. diameter x 28 in. long XAD-2 resin columns by means of a low pressure positive displacement type pump. The column was then washed with water and the effluent continually monitored with a UV detector connected to a strip chart recorder. When the UV absorbance dropped to 25 percent full scale (2000 ml) the water wash was stopped and methanol was then used to elute the absorbed substances. The water wash was discarded. The methanol eluate was flash evaporated at 50° and the residue dissolved in methanol (15-20 ml) and stored at -75° until used. The XAD-2 column was then regenerated by washing with 500 ml methanol: HCl (90 percent:1.0 N) and finally with 2000 ml of water. When not in use, the column was washed with 1 percent Na_2CO_3 to prevent bacterial or mold growth.

GENERATED FREE RADICALS (GFR)

0.5 ml of XAD-2 urine extract was dried under vacuum and the residue dissolved in 1.0 ml of methanol. To this solution were added 1.0 ml of chloroform and 0.1 ml of tetraethyl ammonium hydroxide, and the solution was mixed. A quartz ESR flow cell was filled with the sample so as to avoid trapping of air bubbles. The free radical signal was measured with a Varian spectrometer with special sensitivity modifications.[6] The constant instrumental parameters used were: field selector, 3380 gauss; sweep range, 250 gauss; sweep time, 2.5 minutes; Klystron tuning, 9.65; phase tuning, 2.2; bridge attenuation, 1.9; crystal setting, V=1.0 and H=2.15. For quantification, the peak-to-peak distance in terms of recorder chart divisions were corrected for modulation amplitude, signal level, battery amplifier gain, vertical display, and the number of scans. A unit of ESR was arbitrarily defined:

$$\text{peak-to-peak (chart divisions)} \times \frac{1000}{\text{S.L.}} \times \frac{150}{\text{B.G.}} \times \frac{\text{V.D.}}{64} \times \frac{4}{\text{No. Scans}} .$$

In preliminary experiments, it was found that the GFR signal reached a maximum in about 10 minutes and then decayed slowly over a period of 30-60 minutes. To compare the ESR signal level of the various urine extracts,

the ESR spectra were measured after 5, 10 and 20 minutes to confirm the 10-minute maximum. The 10-minute value then was used to calculate the ESR units present in the sample.

Lipid Peroxides

The lipid peroxides were determined in whole urine by the method of Baker and Wilson.[7] Routinely the $A_{\lambda 525\ nm}$ of the reaction mixture was corrected for the absorbance of a urine blank; ie, reaction mixture without thiobarbituric acid. For quantification, the $A_{\lambda 525\ nm}$ for the reaction mixture was arbitrarily defined as units of lipid peroxide.

Phenolic Acids

The phenolic acids were determined by the method of Bray and Thorpe[8] using caffeic acid as a standard. For quantification, the phenolic acid concentration was expressed in terms of μmoles of caffeic acid.

RESULTS AND DISCUSSION

The rationale for the use of the three-dimensional plot of 24-hour output of urinary phenolic acids, lipid peroxides and GFR was based on preliminary findings with a small number of test subjects.[4] These studies showed that the controls (4 subjects) fell into the boxed volume of the three-dimensional graph (figure 1) as indicated by the solid lines. In this early study the values obtained with stressed subjects, especially coronary artery diseased patients, fell outside this volume suggesting that this three-dimensional representation was a possible bioindicator of the pathological effects of both physical and mental stress; therefore, warranting further testing for possible use in the field. With this in mind, larger population groups of controls and stressed subjects were tested for the application of this procedure to differentiate the various groups from each other. In addition, a number of control subjects were tested on different days to determine individual variations in excretion of the three substances under test.

Aliquots of 24-hour urine collections from each individual subject were analyzed directly for phenolic acid and lipid peroxide content by the methods described in the section titled "Experimental." For the GFR content aliquots of the methanol eluate from XAD-2 chromatography of individual urines were analyzed by ESR for free radical content. The data obtained were then corrected for the dilutions used in the analyses and then listed in tables I, II and III in terms of 24-hour output for phenolic acids (table I), lipid peroxides (table II) and GFR (table III). The tables labelled "A" list the data for acceleration stressed subjects and the "B" tables list the data for the cardiac bypass patients. In addition, the mean value and standard deviation for each test group were calculated and also listed in the respective tables. The mean values and standard deviation for each test group were also plotted as a bar graph in figures 2, 3, and 4. Finally the mean

values for all controls, all acceleration stressed, all preoperative cardiac patients, and post-operative cardiac patients were calculated and plotted in the three-dimensional manner (figure 1).

The 24-hour urinary output of phenolic acids, lipid peroxides and GFR shown in table I reveals large variations in individual values within each test group. Further, in a few controls in which urine collections were obtained on different days, large variations were also found for each individual. As a result, the standard deviation of the means are too large so that the mean value obtained for each test group is mathematically non-significant. Comparison of the mean values and standard deviations for each test group (figures 2, 3, 4) reveals large overlapping of values for each parameter, making it impossible to use any one of these parameters as a differentiation index for stress. Further, in the three-dimensional plot (figure 1) the large overlap of mean values and standard deviation also rules out this method as a bioindicator of pathological stress.

Table IA

Acceleration Stress:
Phenolic Acid Content of Urine
(μmoles per 24 hours)

	Controls				Acceleration Stress			
Subject	Exp't 1	Exp't 2	Exp't 3	Exp't 4	Exp't 1	Exp't 2	Exp't 3	Exp't 4
1	801	4256	7410	6983	463	8991	11326	
2		2232	6365			6493	7741	
3		[2] 6768 3354	3003			6348	2610	
4		3583	2271 5101	3990		7339	6218	2429
5				17798 7193				9876
6				17503 10234				23051
7				14281			9208	20894
8[1]				15059				
9		10117		21776		6667		
11	1117				2852			
12	2757				420			
13	1341				1936			
14	1265				9894			
16	1051				1760			
Mean	1388	11440	5470	12760	2890	7168	7420	14060
S.D.	±695	±15500	±1896	±5960	±356	±1090	±3280	±9670

[1] Female

[2] Data in brackets are for urines collected from the same subjects on different days.

Table IB

Coronary Artery Bypass Patients:
Phenolic Acid Content of Urine
(μmoles per 24 hours)

Subject	Pre Oper	Subject	Pre Oper	Post Oper
3[1]	2497	2-1	6234	10527
4	7022	2-2	4630	
5	8843	2-3[1]	11789	
6	14410	2-4	10942	
8	2790	2-5	14293	
9	3197	2-6[1]	18772	7786
11	16119	2-7	15621	20088
12[1]	4801	2-8	9650	
13[1]	3066	2-10		33739
14[1]	21555			
15[1]	3779			
Mean	8007		11490	18036
±	±6500		±47.3	±11722

[1] Female

Table IIA

Acceleration Stress:
Lipid Peroxide Content of Urine
(units per 24 hours)

	Controls				Acceleration Stress			
Subject	Exp't 1	Exp't 2	Exp't 3	Exp't 4	Exp't 1	Exp't 2	Exp't 3	Exp't 4
1	110	90	213	152	83	158	315	
2	233	98	244			103	269	
3		[2] 95 115	155			124	83	
4		186	[1372 34	57		207	158	53
5				[65 245				40
6				[400 109				97
7				294			152	96
8[1]				116				
9		84		255	179	150		
11	1280				154			
12	1790				131			
13								
14								
15					185			
16	1550				124			
Mean	1610	111	187	190	143	148	195	72
S.D.	±480	±38	±50	±120	±38	±39	±94	±29

[1] Female

[2] Data in brackets for urines collected from the same subject on different days.

Table IIB

Coronary Artery Bypass Patients:
Lipid Peroxide Content of Urine
(units per 24 hours)

Subject	Pre Oper	Subject	Pre Oper	Post Oper
3[1]	430	2-1	394	202
4	389	2-2	153	
5	201	2-3[1]	200	
6	90	2-4	260	
8	101	2-5	220	
9	150	2-6[1]	149	179
11	388	2-7	204	331
12[1]	319	2-8	173	
13[1]	148	2-10		345
14[1]	126			
15[1]	75			
Mean	220		219	264
S.D.	±135		±80	±86

[1] Female

Table IIIA

Acceleration Stress:
Generated Free Radical Content of Urine
(ESRU per 24 hours)

	Controls				Acceleration Stress			
Subject	Exp't 1	Exp't 2	Exp't 3	Exp't 4	Exp't 1	Exp't 2	Exp't 3	Exp't 4
1	254	3840	1717	748	113	3972	2224	
2		1056	1454			1188	1354	
3		[1320 1188	936			1740	367	
4		2940	[896 220	3370		14820	867	5240
5				[3703 1140				8270
6				[2209 1524				2544
7				3576			2604	1408
8[1]				1306				
9		4764		4841		4260		
11	176				310			
12	184				1340			
13	397				1351			
14	162				1634			
Mean	235	2518	1176	2154	751	5196	1480	1321
S.D.	±97	±1570	±378	±1540	±690	±515	±930	±891

[1] Female

Table IIIB

Coronary Artery Bypass Patients:
Generated Free Radical Content of Urine
(ESRU per 24 hours)

Subject	Pre Oper #1	Subject	Pre Oper #2	Post Oper
3[1]	262	2-1	788	
4	480	2-2	0	589
5	880	2-3[1]	1137	
8	219	2-4	1411	
9	208	2-5	1972	
11	1453	2-6[1]	611	754
12[1]	3148	2-7	1220	2889
13[1]	1735	2-8	201	
6	533	2-10		1146
14[1]	1389			
15[1]	373			
Mean	971		918	1320
S.D.	±904		±650	±1007

[1] Female

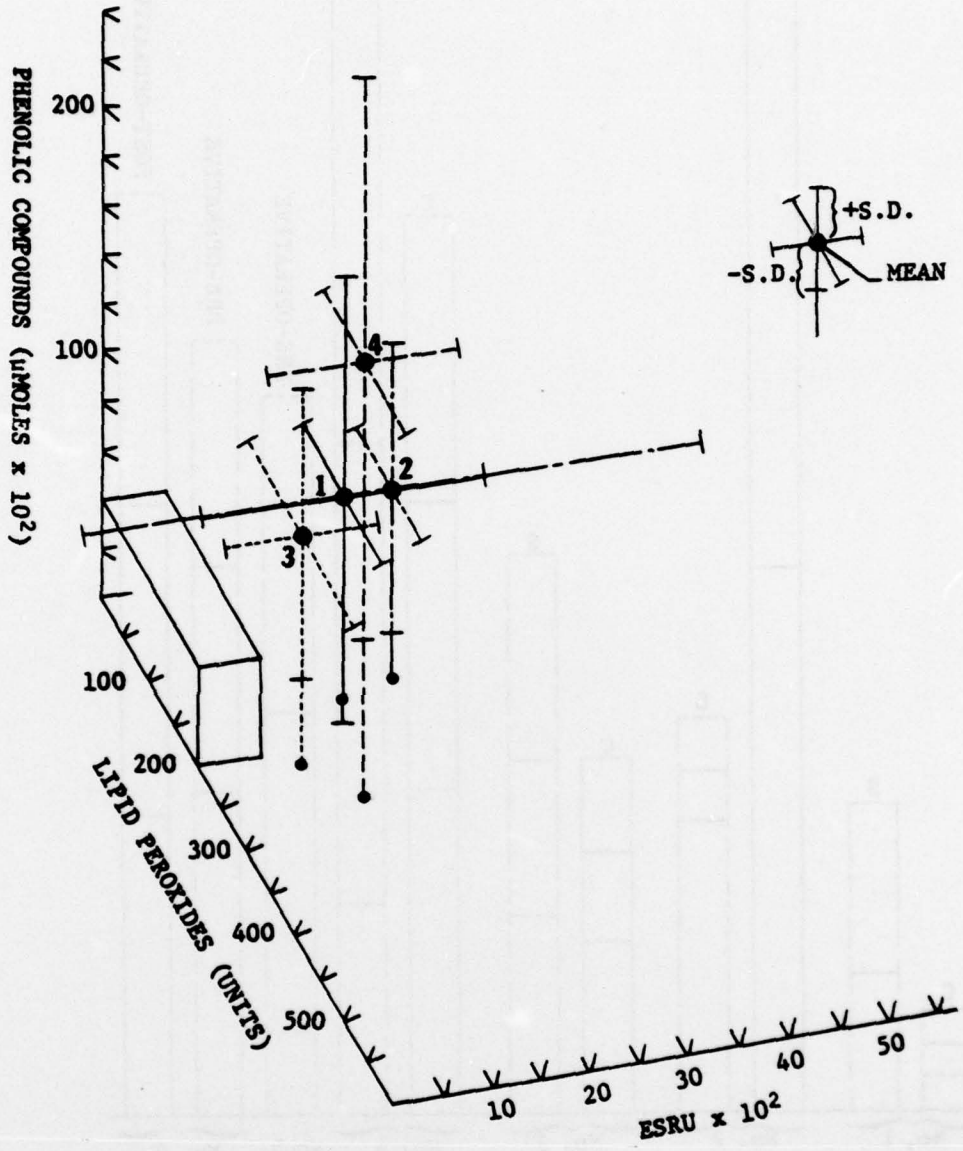

Figure 1 - Three Dimensional Plot of Phenolic Acids, Lipid Peroxides and Generated Free Radicals in the Urines of Control and Stressed Subjects.

The data for each test group was plotted as the mean value ± one standard deviation of each parameter. The solid block volume represents the data spread for four control subjects and five acceleration stressed subjects ( ). Group 1 (——), all control subjects; group 2 (— - —), all acceleration stressed subjects; group 3 (----), all pre-operative cardiac by-pass patients; and group 4 (— — —), all post-operative cardiac by-pass patients.

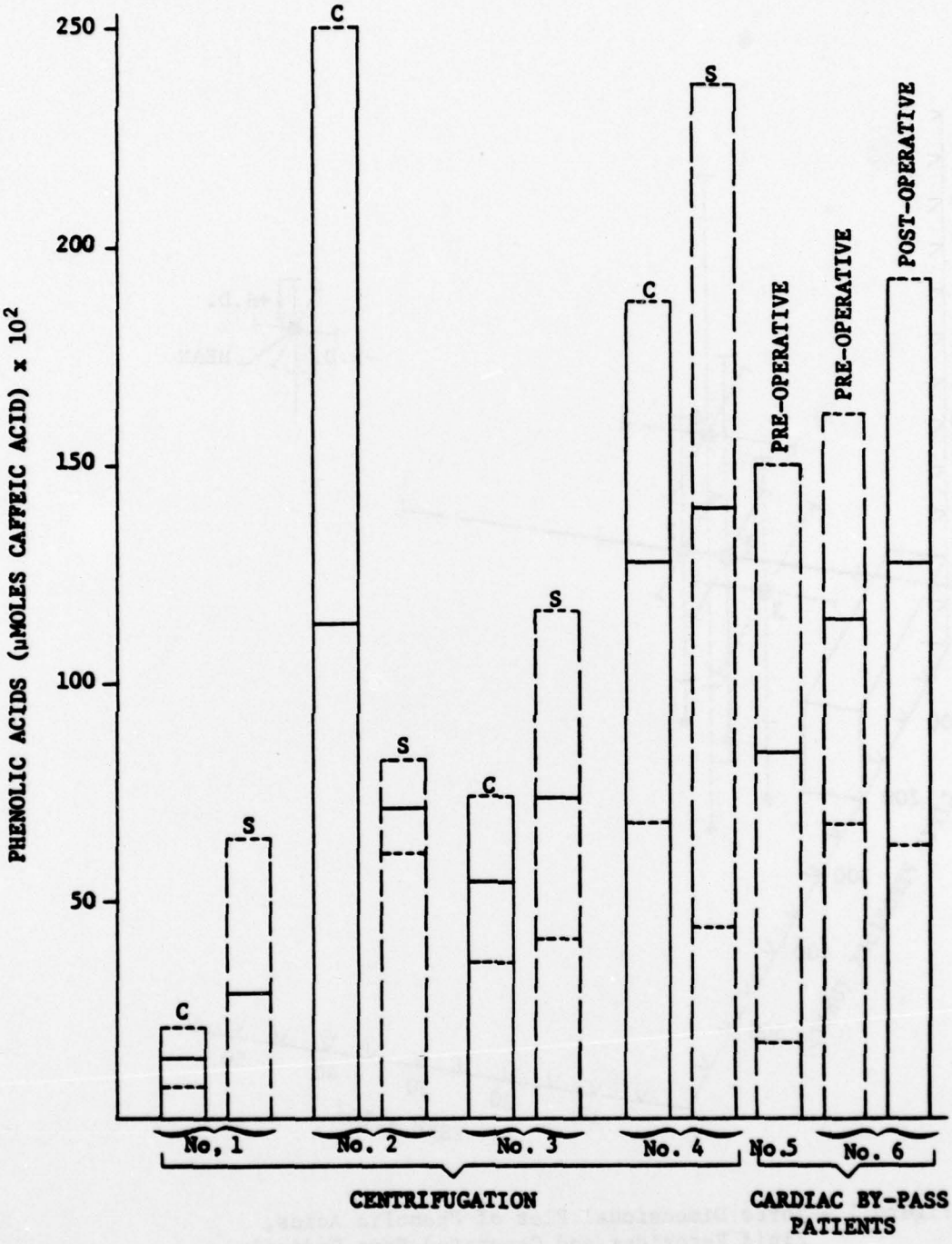

Figure 2 - 24 Hour Urinary Output of Phenolic Acids.

The 24-hour urinary output of phenolic acids from control individuals (C), acceleration stressed individuals (S), and patients for cardiac bypass surgery. The solid horizontal lines are the mean value for each group. The dashed horizontal lines are plus and minus one standard deviation.

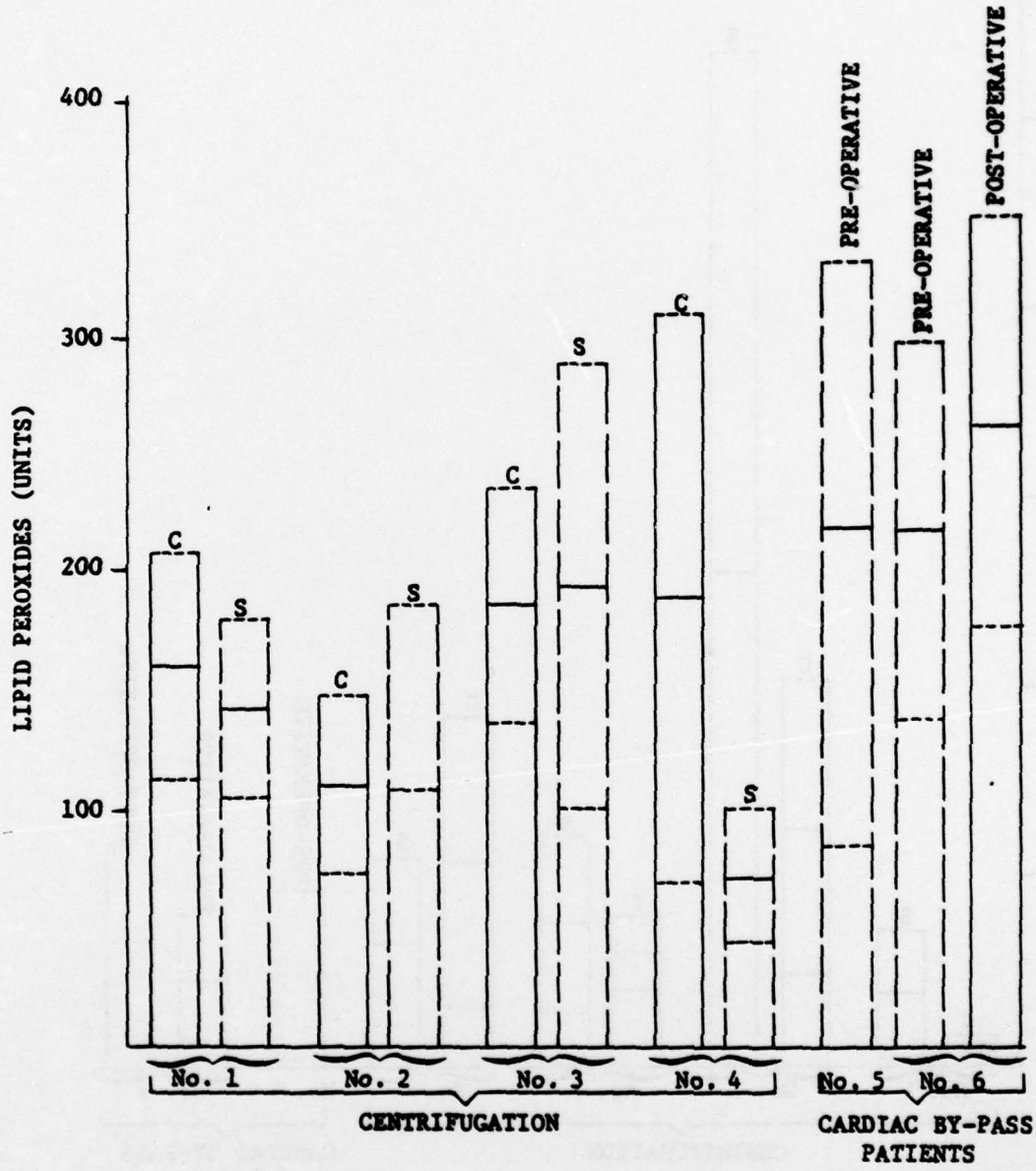

Figure 3 - 24 Hour Urinary Output of Lipid Peroxides.
(See figure 2 for details)

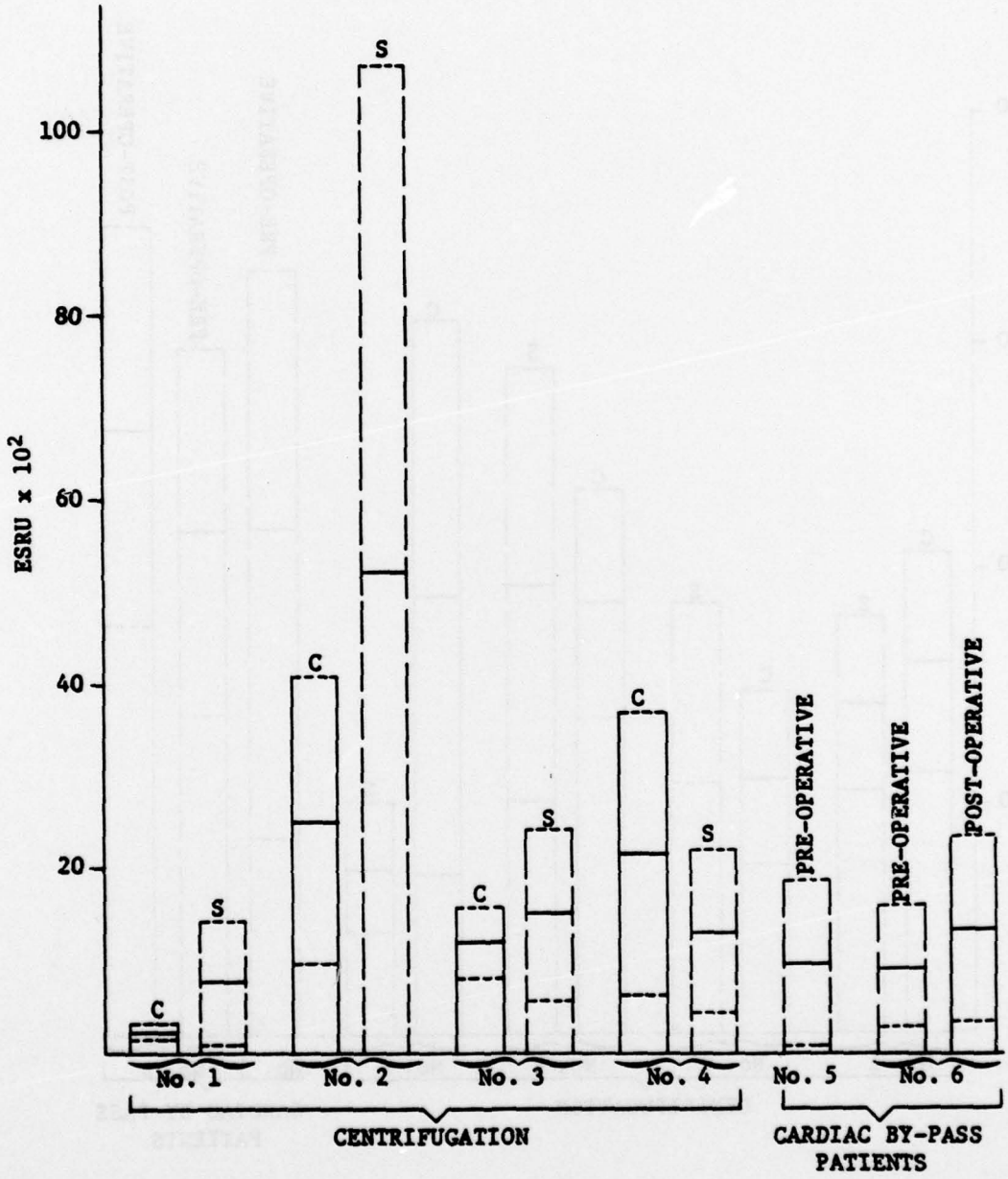

Figure 4 - 24 Hour Urinary Output of Generated Free Radicals.
(See figure 2 for details)

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